Review article

Bulk production of the antiviral lectin griffithsin

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Summary

Application of plant-based protein expression systems for bulk production of recombinant protein pharmaceuticals is building momentum. There are considerable regulatory challenges to consider in commercialization of plant-made pharmaceuticals (PMPs), some of which are inherent to plant-production systems and others that are common with other production systems, but are new to PMPs because of the youth of the industry. In this review, we discuss our recent and ongoing experience with bulk production of the HIV microbicide candidate, griffithsin (GRFT), utilizing plant-based transient protein expression, with specific focus on areas relevant to commercial manufacturing of bulk GRFT active pharmaceutical ingredient (API). Analytical programs have been developed for the qualification and monitoring of both the expression vector system and the API detailing our experience and plans for each. Monitoring postpurification protein modifications are discussed in relation to stability and safety programs. Expression, processing and analytics programs are associated with increased manufacturing costs in current good manufacturing practice (cGMP) production because of the required qualification testing. The impact of these costs on the overall cost of goods is particularly relevant to GRFT manufacturing because GRFT, as an HIV microbicide, is most needed in populations at high risk for HIV exposure in resource-poor countries. Consequently, GRFT for microbicide applications is a very cost-sensitive recombinant PMP. We have therefore emphasized maintaining a low cost of goods. We provide a review of the literature on the economics of PMPs with various expression systems and how they may impact production costs and complexity.

Introduction

Griffithsin (GRFT) is a 121 amino acid lectin identified in the red marine alga, *Griffithsia* sp. (Mori *et al.*, 2005). Structural characterization of GRFT showed it to be a domain swapped homodimer with six carbohydrate binding sites that bind to terminal mannose residues on N-linked glycans of the HIV envelope protein, gp120 (Moulaei *et al.*, 2010; Xue *et al.*, 2012, 2013; Ziolkowska *et al.*, 2006). Subsequent functional characterization of GRFT has demonstrated it to be a potent HIV inhibitor with minimal toxicity *in vitro* and *in vivo* (Barton *et al.*, 2014; Emau *et al.*, 2007; Kouokam *et al.*, 2011; Mori *et al.*, 2005; O'Keefe *et al.*, 2009). GRFT displays picomolar EC_{50} values in its inhibition of laboratory and primary HIV-1 and HIV-2 isolates and has shown stability and activity in macaque vaginal fluid (Emau *et al.*, 2007; Ferir *et al.*, 2012; Mori *et al.*, 2005). Further investigation of GRFT's safety profile revealed that although it can bind the human squamous epithelial layer and peripheral blood mononuclear cells, cells and tissues did not exhibit significant inflammatory responses when exposed to GRFT and GRFT retained picomolar levels of antiviral activity (Kouokam *et al.*, 2011). Our studies on topical delivery of GRFT in a murine model of SARS-Coronavirus infection showed GRFT treatment to be both safe and efficacious (O'Keefe *et al.*, 2010), and studies on systemic delivery of GRFT also showed a good safety profile in rodent models with minimal changes in spleen and liver size and no coincident pathological changes

(Barton *et al.*, 2014). GRFT's favourable safety profile and potent, broad spectrum antiviral activity has promoted its development as an HIV microbicide (Zeitlin *et al.*, 2009).

Development of GRFT as an HIV topical microbicide requires a production system for making bulk quantities of high-quality GRFT available for formulation and characterization. GRFT has been expressed using multiple recombinant systems including *E. coli* and plant systems (Giomarelli *et al.*, 2006; Hahn *et al.*, 2015; O'Keefe *et al.*, 2009; Vafaee *et al.*, 2014). Expression of GRFT using *E. coli* systems has been performed with modest success, where cultures grown in shake flasks were able to produce 12 mg of soluble GRFT per litre, but the majority of the protein formed insoluble inclusion bodies. Optimization of *E. coli* expression systems using fermentors allowed production of >500 mg of GRFT per litre of media (Giomarelli *et al.*, 2006). Initial investment in fermentor systems represents a significant upfront manufacturing cost and necessitates removal of bacterial endotoxin. As an algal protein, GRFT is an ideal candidate for plant-based expression. Recently, GRFT was demonstrated to be stably expressible in chloroplasts of *Nicotiana tabacum* (Vafaee *et al.*, 2014), but more commonly GRFT has been expressed transiently in *Nicotiana benthamiana* using *Agrobacterium*-mediated expression systems or recombinant tobacco mosaic virus (rTMV)-based expression systems (Fuqua *et al.*, 2015; Hahn *et al.*, 2015; O'Keefe *et al.*, 2009; Vafaee *et al.*, 2014). The largest body of published work on manufacturing-scale GRFT production has

been performed using rTMV in *N. benthamiana* with reported *in planta* expression levels ranging from of 0.5–1.0 g/kg fresh weight of plant material, and purification methods yielding 30–60% of total expressed GRFT. We recently reported our efforts to optimize the original purification process for bulk manufacturing of GRFT from laminar tissues of *N. benthamiana* using rTMV-based expression vectors; our new process was able to drastically improve the recovery of GRFT yielding 60–90% of total expressed GRFT (Fuqua *et al.*, 2015; O'Keefe *et al.*, 2009). The updated purification method was piloted at kilogram quantities of plant biomass to assure a robust and relatively simple production methodology was established for GRFT prior to applying cGMP methods for eventual clinical application (Fuqua *et al.*, 2015). In combination, the yield, stability and established purification and analytical protocols applied for manufacturing GRFT provide a valuable example for understanding the complexities and challenges of PMPs at industrial scale, towards developing a plant-made HIV microbicide.

One of the primary challenges in moving from laboratory scale production to bulk production is the regulatory requirements involved with the manufacturing process and protein product qualification. There are few therapeutic proteins produced in plant systems that are approved by regulatory authorities and none of these is produced in leaf tissues. A regulatory framework for the production of recombinant pharmaceuticals in plants only existed in draft form until little more than a decade ago. Moreover, as the industry was still in its infancy, the draft guidance was based on existing knowledge and experience with regulation of pharmaceutical production in mammalian and microbial cells and was lacking in specifics that related to the use of whole plants. In principle, good manufacturing practice (GMP) should be able to be extrapolated from mammalian and microbial technologies to plant-based technologies. In practice, this may work well for products produced in plant cell culture. Whole plant-production systems, however, introduce new and separate challenges (Fischer *et al.*, 2012). This situation has caused the development of two divergent production pathways: plant cell culture systems and whole plant systems. Plant cell culture technologies have the advantage of allowing enough similarity in concept and process to mammalian and microbial expression systems to have a clearer and more straightforward regulatory pathway. Facilities using whole plant technologies had to work with regulators to make clear the requirements for handling new issues, such as seed banking instead of cell banking (Fischer *et al.*, 2012). Therefore, mechanisms for process qualifications for plant expression must be developed from a thorough understanding of regulatory requirements. In this review, we will discuss the regulatory aspects involved in the expression of GRFT using rTMV technology and outline our plan for qualification of virion and plasmid banks including long-term stability monitoring.

The requirement for qualification of drug substance is common to all technologies used for protein production. Although the regulatory path for PMPs versus mammalian, insect cell, or microbial systems may exhibit some difference, the basic tenets of GMP hold true across the board: All pharmaceuticals are required to be manufactured and released according to cGMP. In an effort to provide guidance for future PMPs, we have included the certificate of analysis (CofA) for GRFT and a stability plan with an overview of techniques applied to assess product stability. In this review, we specifically discuss protein oxidation as a potential issue when producing and assessing the long-term stability of biologics because protein oxidation can have detrimental impact on activity, stability and safety (Singh, 2011). Of course, oxidation is only one of many potential product degradation pathways that must be considered, but oxidation has been raised as a potential concern for GRFT because the cervicovaginal compartment and to a lesser extent the anal compartment, both of which are potential routes of administration for GRFT-containing microbicides, maintains hydrogen peroxide producing bacteria (Zegels *et al.*, 2010). With such potential for oxidation upon delivery, it is necessary to monitor oxidation at the level of the active pharmaceutical ingredient (API), formulated product, and in subject samples after delivery. This review is focused on the production and quality characterization of GRFT and therefore will focus on the API. We have provided our contingency plans and reasoning for dealing with protein oxidation in the event GRFT demonstrates oxidation issues. Our first product that we will evaluate in the clinic is a topical formulation designed for rectal use. We have not yet submitted the investigational new drug (IND) application to FDA, so cannot say definitively that what we present here is acceptable to the agency, but this article details some of our regulatory efforts to date. Therefore, the authors request that the regulatory position taken in this article be read as opinion and not as practice known to be acceptable to FDA.

As an HIV microbicide, GRFT is targeted for use by individuals at high-risk for HIV exposure. Some of the most at-risk HIV populations are found in resource-poor countries, and in poor communities in developed countries. Therefore, cost of goods with GRFT-based microbicides will be a very sensitive issue. Reducing the production costs and in turn reducing the price per dose of GRFT will expand the availability of GRFT in areas with the highest HIV burden. To this end, we need a better understanding of the production costs of GRFT. Many theoretical studies have been performed examining the cost of goods of multiple products to demonstrate the economic feasibility of PMPs, all of which show that the economics is product, process and scale dependent (Tuse *et al.*, 2014; Walwyn *et al.*, 2015). Success of GRFT-based commercial products is dependent on its performance in preclinical and clinical trials (safety and efficacy); however, practical issues regarding its availability as a sexual health product to a substantial population of potential users is contingent on the cost, which is driven in part by the production process. Therefore, continued optimization of the production process may be necessary. Certainly, the purification process has been improved tremendously by our group (Fuqua *et al.*, 2015). Further optimization of these technologies will need to be undertaken with economics as a primary concern. We put forth, in this review, potential expression systems to be tested, and our opinions of the economic impact process changes may have on the cost of goods. We acknowledge that successful development of GRFT as an HIV microbicide will depend solely on its clinical performance as long as hurdles related to bulk PMP production are overcome with proper foresight.

Development and qualification of plasmid and virion banks for GRFT production

Our experience with manufacturing-scale production of GRFT primarily has used dual-subgenomic promoter rTMV as the transient expression system (Fuqua *et al.*, 2015; McCormick *et al.*, 1999; O'Keefe *et al.*, 2009; Shivprasad *et al.*, 1999); it has proven to be scalable with few process-related issues. Transient expression of GRFT in *N. benthamiana* using rTMV can be done using naked infectious RNA transcribed from the plasmid or using first passage virus particles isolated from RNA-infected plants. At
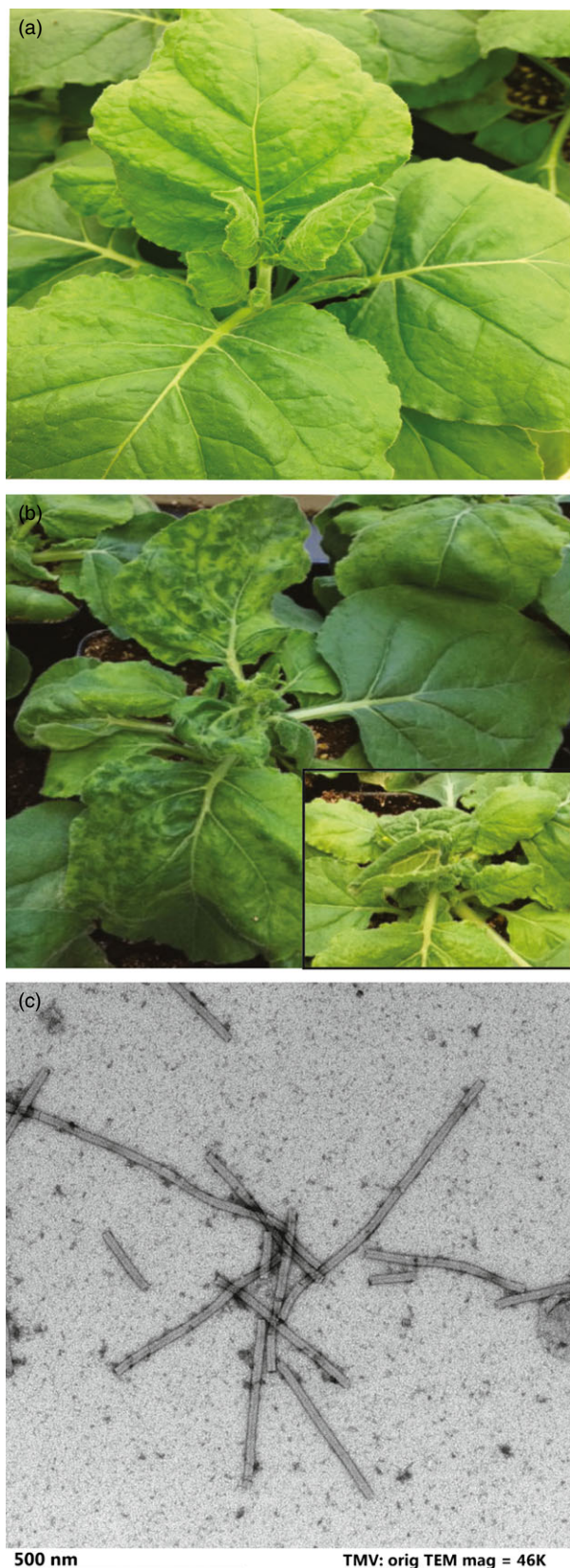
Figure 1 GRFT expressing plants and infectious rTMV particles. (a) Plants not expressing GRFT or infected with rTMV show no visible symptoms of TMV infection. (b) Plants expressing GRFT using rTMV shows leaf mottling, growth retardation and deformation of the leaves. The inset picture is a close-up of the visible growth deformities, with a curling of the leaves and significantly reduced size of infected leaves. (c) The purified rTMV virion used for GRFT expression was imaged by transmission electron microscopy under 46K magnification after uranyl acetate staining. Intact full length virions can be confirmed with a minimal number of partially formed particles.

genome of the rTMV and continued assessment of the plasmid and virion for the duration of the manufacturing process. We have developed a program to monitor the stability and quality of the virion over the life of GRFT production, which will provide stability and activity data after long-term storage. The origin–plasmid DNA contains a dual-subgenomic promoter of infectious TMVU1 sequences upstream of the coat protein promoter behind which a *N. benthamiana* codon-optimized sequence of GRFT was cloned. The rTMV vector, designed by Shivprasad *et al.*, used the heterologous tobacco mild green mosaic virus (U5) 3′ sequences, coat protein cistron and 3′ UTR (Figure S1). The origin–plasmid was sequenced in both 5′ and 3′ directions, and a large amount of origin–plasmid DNA was purified using an endotoxin-free plasmid prep. The resulting purified plasmid was aliquoted and deemed the 'plasmid bank'. The plasmid bank was qualified by double-strand sequencing and comparison to the origin–plasmid. Ability to produce virion particles and GRFT from plasmid bank expression is assessed *in planta* by transcribing the DNA to RNA using an RNA transcription kit and subsequently infecting plants with the naked RNA (McCormick *et al.*, 1999, 2003; O'Keefe *et al.*, 2009). Infected plants are inspected for TMV-related symptoms at the specified harvest time with the qualification metric requiring 90% of inoculated plants to show visible symptoms (Figure 1a and b). Plants are harvested, and a gp120-capture enzyme-linked immunosorbent assay (gp120 ELISA) was used to quantify the produced GRFT protein in the green juice homogenate (O'Keefe *et al.*, 2009). For qualification of the inoculum, the infected plants have to be producing GRFT in excess of 200 mg/kg of biomass. Failure of plasmid to match the origin sequence or failure of the transcribed plasmid to induce significant symptomology in inoculated plants or failure to reach the target expression concentration of GRFT disqualified the plasmid bank in its entirety, and requires re-creation of the bank.

After production and initial qualification, the plasmid bank is stored at −20 °C and placed on a stability monitoring program (Table 1). The stability monitoring program for the plasmid bank includes monitoring sequence stability and functionality. Sequence stability is monitored by double-strand sequencing the GRFT sequence within the plasmid, and functionality is monitored by transcribing the plasmid and assuring the naked TMV RNA remains infectious, using the infectivity metric of symptomology. It is assumed the plasmid bank will remain stable for >2 years, but this needs to be validated empirically using the proposed methods to understand if and when the plasmid bank might fail.

After successful production and qualification of the plasmid bank, it is used to transcribe infectious RNA for inoculation of a number of plants, which is determined based on the size of the bank you are producing (the final virion volume will be approximately 10% of the harvested biomass). The plants are harvested relatively early in the normal production process, approximately

scale, the most cost-effective and reproducible method is to use first passage virions. Production of TMV virions for manufacturing requires qualification of the origin–plasmid containing the

Table 1 GRFT Plasmid and Virion Bank Stability Plan. At each time point, the assays will be performed on a fresh vial of GRFT virion and plasmid stored at −20 °C

GRFT plasmid and virion bank stability plan

	Year 1 (Month)						Year 2	
	0	1	3	6	9	12	18	24
Plasmid bank stability								
Double-strand Sequencing of Plasmid	●							
GRFT DNA Sequencing within Plasmid		●		●		●		●
Symptomology from Transcript	●	●		●		●		●
GRFT Expression from Transcript	●	●		●		●		●
Virion bank stability								
Absorbance (OD$_{260}$)		●	●	●	●	●	●	●
Symptomology		●	●	●	●	●	●	●
GRFT Expression		●	●	●	●	●	●	●

day 5 after infection. The virion amplified in the plant tissue is extracted, isolated and concentrated using a modified polyethylene glycol precipitation procedure and stored at −20 °C (Asurmendi *et al.*, 2007). An approximate concentration of the virion can be determined through UV absorption at 260 nm with an extinction coefficient of 3.0 (Kathiria *et al.*, 2010). The isolated virion is qualified for use through functional assessment by application of 50 ng of virus per plant with a mild abrasive (Kathiria *et al.*, 2010). Function is assessed by display of symptoms characteristic of rTMV vector infection and GRFT expression level with the same criteria required as with 'naked' RNA, 90% symptomatic plants and >200 mg/kg of GRFT. For comparison to prior work with TMV, electron microscopy images were used to visualize TMV particles and data were compared to historical information regarding TMV virion size (Figure 1c). Upon qualification of the virion bank, a stability program will be implemented (Table 1). The stability program mirrors the qualification program, but is carried out over a 2-year period as the virion bank is being stored at −20 °C.

The inoculum stability program discussed herein can be used for other PMPs expressed through rTMV systems. Once the 2-year stability program is complete, we will have a better understanding of the long-term viability of the plasmid and virion banks.

Protein qualification and stability monitoring

Being cognizant that problems in method scalability and technology transfer are inevitable when changing from a research to manufacturing-orientated setting, we optimized purification processes for GRFT at pilot production scale, in the Kentucky Bioprocessing, Inc. (Owensboro, KY, USA) facility where cGMP manufacturing will occur, to enable seamless scale-up of the process. Pilot scale production assessing feasibility and limitations of processes is important to understand yield and properly plan scale-up studies. Process optimization is therefore a necessary initial step in bulk production prior to developing the documentation and analytics necessary for proper cGMP production. cGMP requires the development of validated product release assays that prove acceptability of critical product parameters of identity, purity, potency and safety. Quality is established through char-

acterization and stability testing with quality parameters established through repeated measurements on multiple batches. The current release criteria for GRFT include visual appearance, molecular weight determined by mass spectrometry, pH determination of the solution, protein concentration determined by OD$_{280}$, purity determined by SDS-PAGE and size-exclusion high-performance liquid chromatography (SEC-HPLC), potency determined by a gp120 ELISA, safety determined by endotoxin levels and bioburden and residual TMV determined by infectivity tittering in a local lesion host (*N. tabacum* cv Glurk). Decisions regarding acceptable bioburden and endotoxin levels are informed by dosage form, dosage regime and route of administration. FDA guidance states that the calculation methods described in United States Pharmacopeia monograph 85 (USP <85>) or Association for the Advancement of Medical Instrumentation (AAMI) standards are appropriate for establishing the endotoxin limit for a product (US Department of Health and Human Services Food and Drug Administration, 2012). Unfortunately, there are no specific benchmark standards or guidances for bioburden for rectal product administration that are provided by any regulatory agency (although a USP chapter is proposed at this issue). USP guidelines in Chapter 1111, 'Microbiological Attributes of Nonsterile Pharmaceutical Products', do not provide specific guidance for topical preparations, but do specify that the 'significance of microorganisms in nonsterile pharmaceutical products should be evaluated in terms of the use of the product, the nature of the product, and the potential hazard to the user' (United States Pharmacopeia Convention, 2013). It is helpful to note that the maximum total aerobic microbial count (TAMC) for a rectally administered product specified in both the World Health Organization's International Pharmacopoeia as well as the European Pharmacopoeia is 10^3 CFU/G, CFU/ML; the maximum total combined yeasts and moulds count (TYMC) is 10^2 CFU/G, CFU/ML (European Directorate for the Quality of Medicines & Healthcare, 2015) (World Health Organization, 2014). The rectum is a notably nonsterile biological compartment. Testing is therefore included in the rectal GRFT CofA for information only and is likely not an actual acceptance criterion.

Table 2 details the current quality profile for GRFT bulk API and will be used for the release of GRFT manufactured under cGMP. Stability testing includes some methods not required for release but used for information only. Common methods include OD$_{280}$ and SEC-HPLC and potency ELISA to monitor the physical (degradation and aggregation) and functional stability (i.e. gp120-binding affinity), respectively. To be a viable microbicide product, GRFT must have a shelf life of at least 2 years. Therefore, we have designed a 2-year stability program (Table 3) to monitor the chemical, physical, thermal and functional stability of GRFT. Specifically, oxidation, aggregation, degradation, melting temperature and potency of GRFT will be evaluated. Currently, we have completed 3 months of stability including GRFT API stored at room temperature and 4 °C. There was no detectable change in any measured aspect of the GRFT API. Figure 2 highlights some of the data generated at the 3 month stability time point. A SEC-HPLC method has been developed as a standard operating procedure (SOP) for the product release of GRFT as well as to monitor the physical stability. This SOP is being transferred to Kentucky Bioprocessing, Inc., a facility capable of large-scale cGMP production of GRFT, with the assay criteria that the GRFT native dimer must show >95% purity and the retention time of the dimer must be within 95% confidence interval of reference standard GRFT from three independent runs. SEC-HPLC is a

Table 2 Certificate of Analysis (CofA) for the Manufacturing Release of GRFT. The CofA contains the methods and parameter tested as well as the specifications that need to be met for release

Certificate of analysis
GRFT API

Lot no.		Date of manufacture
Test Parameter	Test Method	Specification
Protein Concentration	UV Absorbance (A_{280})	≥10 mg/mL
Purity	Reducing SDS-PAGE	≥95% GRFT monomer
Purity	Size Exclusion HPLC	≥95% GRFT dimer
Potency	gp120 ELISA	EC_{50} – 5–50 ng/mL
Appearance	Visible Appearance	Clear, Colourless to Amber, Liquid, Free of Visible Particles
Identity	Mass Spectrometry	12731.9 ± 6 Dalton
Physical/Chemical Properties	pH Determination	7.2 ± 0.2
Safety	Endotoxin	<5 EU/mL
Safety	Bioburden	Report Result (For Information Only)
Impurity	Glurk Assay	Report Result (For Information Only)

Table 3 GRFT Stability Plan. At each time point, the assays will be performed on an unopened vial of GRFT stored at room temperature and 4 °C. Parentheses (●) represent optional assays that will be performed if changes in other required analytical end-points is observed

GRFT stability plan

	Year 1 (Month)						Year 2	
	0	1	3	6	9	12	18	24
Chemical stability								
Oxidation	●	●	●	●	●	●	●	●
Physical stability								
Absorbance (OD_{280})	●	●	●	●	●	●	●	●
Size exclusion HPLC (SEC-HPLC)	●	●	●	●	●	●	●	●
Reducing SDS-PAGE	●	●	●	●	●	●	●	●
Thermal stability								
Differential scanning fluorimetry (DSF)	●	●	●	●	(●)	●	(●)	●
Functional stability								
gp120 ELISA	●	●	●	●	●	●	●	●
Surface Plasmon Resonance	●	●	●	●	(●)	●	(●)	●

dynamic method to show the purity of GRFT API as well as to monitor the aggregation and degradation of GRFT. Figure 2a shows that GRFT is >95% pure, and after 3 months of shelf life stability assessment, there is no detectible aggregation or degradation of GRFT. Another analytical assay we have developed for GRFT product release and to monitor stability is a potency validation assay, which will ensure the anti-HIV activity of GRFT. Specifically, GRFT's binding to a recombinant HIV-1 envelope glycoprotein gp120 (which is solely responsible for GRFT anti-HIV

activity) will be verified by gp120 ELISA. The data in Figure 2b demonstrate that GRFT is functionally active, binding to gp120 in the nanomolar range. After 3 months, GRFT has not lost potency. To further validate the gp120 binding activity of GRFT, surface plasmon resonance (SPR) was employed to determine the equilibrium dissociation constant (K_d) (Figure 2c). The average K_d of GRFT to gp120 was found to be 31.9 nM and after 3 months, while GRFT stored at 4 °C and room temperature (RT) had K_d values of 32.2 and 33.0 nM, respectively. Thus, there is no significant loss in GRFT binding to gp120 after 3 months of storage. Another parameter to highlight is the thermostability of GRFT. Differential scanning fluorimetry (DSF) was used to determine the melting temperature (T_m) of GRFT (Figure 2d). After 3 months of storage at 4 °C or RT, there was no significant change in the T_m therefore confirming the structural integrity of GRFT. Lastly, although not discussed here, the concentration of GRFT as determined by OD_{280} and the purity of the monomer species monitored by reducing SDS-PAGE were unaltered after 3 months of storage.

Protein oxidation

A critical area of therapeutic protein development related to storage, production and delivery-site, that is often overlooked at the laboratory scale, is amino acid modifications of the protein product including oxidation, deamidation, glycation. Progressive modifications of a protein over time will become increasingly problematic because the identity of the API is not fixed, particularly when such modifications affect product safety and/ or efficacy. Oxidation of a PMP can occur *in planta*, during production, storage or upon contact with biological fluids. A mixed nonoxidized and oxidized product is likely to be seen by FDA as a degradation product or an impurity, and the FDA requires that an impurity be characterized. Presence of oxidized product may also raise concerns regarding immunogenicity (Chirino *et al.*, 2004; Singh, 2011). Propensity of a biopharmaceutical to oxidize could complicate the required pharmacology and toxicology studies, as it may necessitate evaluation of toxicity of the oxidized and nonoxidized products, as well as generation of stability and activity profiles of homogenic and heterogenic products. Oxidative products should therefore be identified and characterized early in the development process to avoid the additional risk, time and economic costs of bridging studies.

There are several options for handling the presence of an oxidized impurity. One option, for handling a mixed product, is to fully oxidize the API. If a fully oxidize product is produced, testing will have to be performed to ensure the product is fully oxidized and does not leave a nonoxidized form as an impurity; that the oxidized product does not alter activity; that the oxidized product does not form aggregates; and that the oxidized product will perform adequately in *in vivo* safety studies.

Rather than fully oxidizing the entire molecule, the API can be protected against oxidation through formulation with excipients designed to inhibit oxidation, or even through site directed mutagenesis of the relevant amino acid residues, assuming no negative impact on product activity or performance. Molecule design is an important factor in addressing heterogeneity in a product, for stability issues as well as improving the immunogenic profile of a product (Singh, 2011). Methionine, cysteine and tryptophan are the most commonly oxidized amino acids, but generally details of protein structure can be used as a guide by identifying exposed amino acids. Protection against oxidation
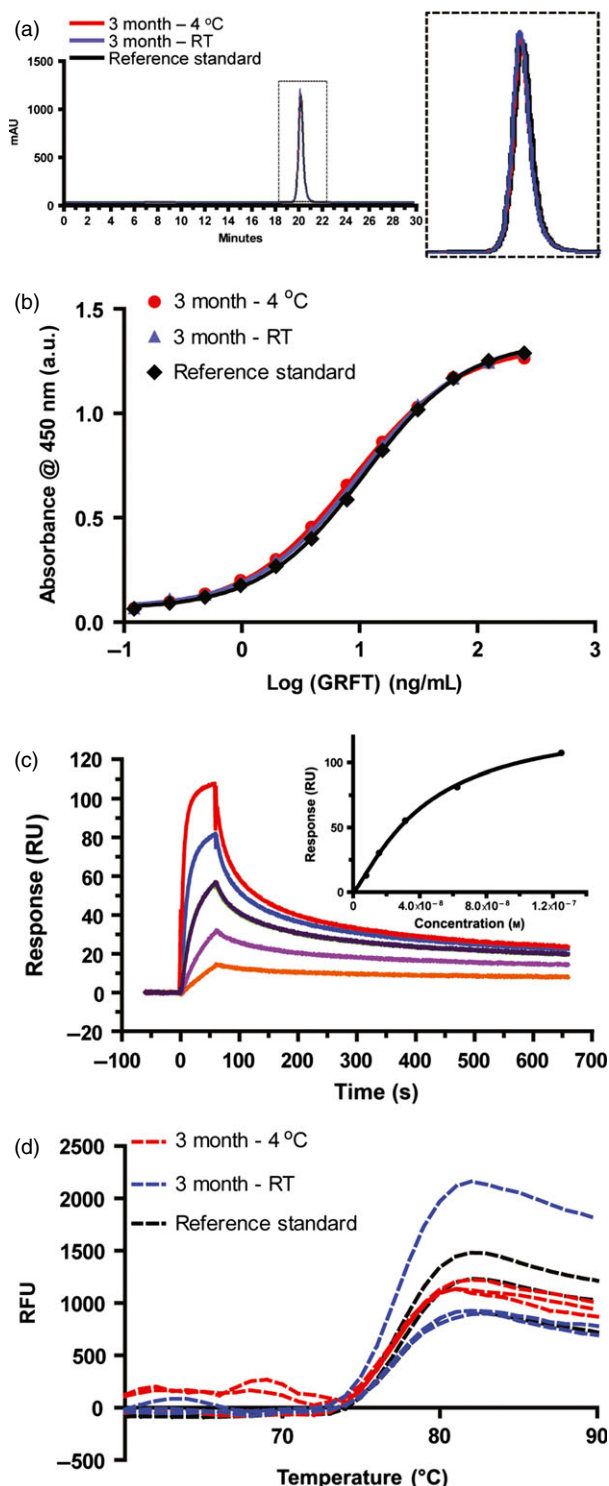
Figure 2 GRFT Stability Profile. GRFT remains stable after 3 months stored at room temperature (RT) or 4 °C. (a) SEC-HPLC. SEC was performed on a Beckman Coulter System Gold HPLC. An aliquot of 10 μL of the final formulation GRFT (4 °C, RT and reference standard; 10 mg/mL) was applied, to an SEC column (TSKgel SuperSW3000, 4.6 mm I.D. × 30 cm, 4 μm; TOSOH Biosciences, LLC; TSKgel guardcolumn SuperSW, 4.6 mm I.D. × 30 cm, 4 μm; TOSOH Biosciences, LLC) equilibrated in running buffer (100 mM sodium phosphate [pH 7.2], 150 mM sodium chloride, 0.05% sodium azide). After injection, running buffer was applied to the column at flow rate of 0.2 mL/min for 30 min. The inset is a zoomed in picture of the GRFT peak. The large peak shows the dimer formation and purity of GRFT. GRFT is >99% pure and with no aggregation after 3 months. (b) gp120 ELISA. The 50% effective concentration (EC_{50}) for GRFT to gp120 was determined employing a gp120 ELISA as previously described (O'Keefe et al., 2009). The log (agonist) vs. response – Variable slope (four parameters) was used to fit the curve and calculate EC_{50} values in GraphPad Prism 5.0 software. The EC_{50} values were determined to be 8.7 ng/mL, 10.1 ng/mL and 10.9 ng/mL for GRFT stored at 4 °C, GRFT stored at RT and reference standard GRFT, respectively. (c) SPR. The equilibrium dissociation constant (K_d) of GRFT to gp120 was measured using a Biacore X100 2.0 instrument at ambient temperature. For each sample (4 °C, RT and reference standard), the assay was performed in triplicate. A representative sensorgram is shown for the reference standard, which was sequestered from the initial GRFT production. Recombinant biotinylated gp120 (Du151, HIV1/Clade C, Immune-tech # IT-001-139p-Biotin) at a concentration of 1 μg/mL was immobilized on a SA sensor chip (Biacore #BR-1000-32) in 10 mM sodium acetate pH 5.5 with a flow rate of 5 μL/min and a contact time of 150 s. Serial dilutions of GRFT (125, 62.5, 31.3, 15.6, and 7.8 nM) were injected, at a flow rate of 5 μL/min, for a contact time of 60 s and a dissociation time of 600 s. Each set of data was analysed using the steady state affinity analysis (Inset) in the Biacore X100 2.0 evaluation software. The K_d values (average ± SD, $n = 3$) were determined to be 32.2 ± 1.6 nM, 33.0 ± 1.3 nM and 31.9 ± 1.8 nM for GRFT stored at 4 °C, GRFT stored at RT and reference standard GRFT, respectively. (d) DSF. The melting temperature (T_m) of GRFT, at a final concentration of 62.5 μM in PBS, was determined on a BioRad iQ5 multicolour real-time PCR system as previously described (Hamorsky et al., 2013). The T_m values (average ± SD, $n = 3$) were determined to be 74.2 ± 3.9 °C, 76.8 ± 0.9 °C and 77.5 ± 0.8 °C for GRFT stored at 4 °C, GRFT stored at RT and reference standard GRFT, respectively.

collected in an effort to preemptively resolve issues related to oxidation of GRFT. Alternative amino acid sequences, reducing the number of oxidizable amino acids, have been developed that would provide oxidation resistance, and formulations addressing oxidation protection are under development. These options are all being developed concurrently as contingencies for oxidation. The need for an alternative API or formulation has not been established in the field, but successful clinical development will require any issues with oxidation be resolved.

Competing technologies and potential economic impacts of PMPs in resource-poor settings

GRFT's use as an HIV microbicide sets a target demographic of individuals who are at high risk of HIV exposure with many of those individuals in resource-poor settings. Therefore, in our analysis of bulk GRFT production, cost is paramount. Plant-production systems have been touted as being economically advantageous as compared to more traditional cell culture production systems. Reasons for this include low upstream costs, ease of scalability and the lack of adventitious animal pathogens.

requires additional excipients in the formulation and adds another layer of complexity, while direct modification of the amino acid sequence requires confirmation of the activity, structural stability, safety and potential modifications to the manufacturing process.

In our work with GRFT, protein oxidation is and continues to be a potential concern because the amino acid sequence contains multiple potentially oxidizable amino acids (Figure 3). Scaled manufacture in conjunction with long-term storage is a variable not generally simulated in the laboratory. Multiple contingency plans have been discussed, and preliminary data have been

Figure 3 GRFT Crystal Structure. The crystal structure of GRFT (PDB ID: 2GTY) is shown highlighting potentially oxidizable amino acids, methionine, cysteine and tryptophan. GRFT homodimer (yellow and green) has methionines at positions 61 and 78 (shown in black) with no cysteines or tryptophans. Images were created in Accelrys Discovery Studio Visualizer 2.5.

Economic advantage, however, has not yet been proven in practice or in published studies to any appreciable extent (Walwyn *et al.*, 2015). Most of the cost of manufacturing proteins is associated with downstream processing, specifically purification and analytics. Downstream costs were not scrutinized carefully in early economic analyses of plant-production systems and are generally estimated to account for 65% to 95% of the cost of goods. An exception may be applied if the product does not involve significant downstream processing. Consideration of upstream and downstream processing costs as independent variables leads to the assumption that optimization of the expression system would have little impact on the cost of goods (Fischer *et al.*, 2013; Wilken and Nikolov, 2012).

The theoretical lack of impact of upstream cost-saving measures on the overall cost of goods has limited their investigation in side-by-side comparator studies of multiple expression systems. However, upstream methodologies can impact downstream processing costs and have a multiplicative effect on the overall cost-savings. Changing the upstream expression methodology could result in significant cost-savings, but have not been investigated because of limitations on time and the necessity for immediate GRFT production. Upstream expression technologies such as *Agrobacterium* infiltration (agroinfiltration)-based transient expression and stable transgenic expression methods could reduce cost by increasing yield per biomass or reducing the complexity of downstream processing. Expression systems requiring reduced biomass processing for similar yield can have a significant cost-saving effect. Buyel and Fischer (2012) demonstrated that reducing the amount of material processed by harvesting specific leaves can have a significant cost-saving impact by reducing the total process volume which reduces downstream processing costs (Buyel and Fischer, 2012). Expression technologies that provide significantly higher yield per biomass have the potential to reduce both upstream and downstream processing. Additionally, expression technologies that reduce the need for downstream processing can have a substantial impact on overall cost of goods. For example, transgenic plants would have a modest impact on the upstream costs by removing the continual need for transient expression systems, but have the potential to

simplify downstream processing by limiting transient expression system-derived contaminants such as TMV coat protein, in rTMV-based systems and endotoxin, in agroinfiltration-based systems. In the cost assessment of PMP products, the upstream and downstream costs are not fixed relative to each other and upstream processes can significantly influence downstream costs. Currently multiple systems can be used to produce GRFT in plants: Agrobacterium-based, rTMV-based, transplastomics, amongst others (Fuqua *et al.*, 2015; Hahn *et al.*, 2015; O'Keefe *et al.*, 2009; Vafaee *et al.*, 2014). Transgenic lines with inducible expression systems provide interesting new potentials for optimizing upstream expression costs (Werner *et al.*, 2011). All of these alternative expression systems would have varying degrees of impact on GRFT's production costs. In targeting GRFT to resource-limited settings we need to reduce its overall cost, but this will require data from multiple expression systems coupled with optimization of purification methodologies to develop proper economic models related to overall cost. To validate economic models related to the cost of GRFT, it will then be necessary to scale the processes and verify the predicted outcomes in head-to-head comparisons at pilot manufacturing scale.

Commercial advancements in PMPs in the U.S. were boosted in 2009 with the investment of tens of millions of dollars in several PMP facilities. Dubbed 'Blue Angel', the Defense Advanced Research Projects Agency (DARPA) investments increased the existence and capacity of full-scale PMP facilities. The availability of these commercial-scale operations may allow greater proof of concept of the putative economic advantage of PMP technologies at large scale. The existence of large-scale facilities should allow for the performance of economic studies to optimize PMPs as well as the ability to provide therapeutics that target resource-poor settings.

In Ma *et al.* (2013) suggested four points to maximize the opportunity of molecular pharming, the first of which was (i) to prioritize drug targets relevant to developing countries. As an HIV microbicide, GRFT meets the criteria of being a drug that is highly relevant to resource-poor countries. The second suggestion put forth was (ii) to develop partnerships in low- and middle-income countries to allow development of local expertise and technology transfer. As GRFT progresses through its current clinical development plan, we will begin exploring potential manufacturing partners in low- or middle- income areas with the heaviest burden of HIV in hopes of establishing 'production in the region, for the region' (Sack *et al.*, 2015). Following the recent example of the Council for Scientific and Industrial Research of South Africa (CSIR) and Kentucky Bioprocessing, Inc., collaborative agreements between established processing facilities and emerging facilities can be used to speed up the commercialization process. Engaging potential partners early in the development process prior to final process optimization should allow seamless technology transfer of products that are resource-poor focused. An additional two points were made in Ma *et al.*, 2013, which were iii) to increase collaboration between regulatory bodies and iv) to promote socially responsible intellectual property management. At this time in the development of GRFT, it is difficult to address these concerns because regulatory bodies have not yet been queried and the intellectual properties rights have already been established, but the overall value of the proposed points in maximizing the significance of plant-made products is evident. However, the success of GRFT as a product and need to produce at bulk scale in developing countries is contingent upon successful bulk

production and positive clinical outcomes under the current infrastructure.

Concluding remarks

The outlook on development of additional PMP products to clinically relevant therapeutics is currently favourable, but this is much more related to positive outcomes in clinical development than a validation of PMPs. GRFT has been shown to be safe and effective in *in vivo* and *in vitro* models and warrants further clinical development. For the continued clinical development of GRFT, a bulk production system is needed and has been developed in parallel to GRFT's clinical development. There are unique challenges associated with the development of every protein biopharmaceutical and the relative immaturity of PMPs, as a bulk production platform, provides additional challenges. Plant systems seem to be optimum for the production of GRFT, which may be related to its inherent stability and natural production in red alga. In this review, we have updated the field on challenges associated with the bulk production of GRFT and the progress made in addressing those challenges. Systems have been developed and are currently in place for the monitoring and use of rTMV-based expression systems. Programs for the qualification and assessment of the long-term stability of GRFT have been developed and implemented. The GRFT API has demonstrated to be stable and robust with no significant changes in purity or activity over 3 months. The potential remains with GRFT rectal microbicide, like many biologics, that molecular heterogeneity and immunogenicity could become an issue in either the API, formulated product or upon delivery. Systems are being developed and preliminary data collected to overcome issues related to the oxidation of GRFT; assessing formulation and direct molecular alteration solutions. Successful development of GRFT as a safe and effective microbicide in the current clinical trials will then necessitate its availability to at-risk populations. The logistics of acquiring enough GRFT to provide it prophylactically to those at high risk for HIV exposure will require increased production infrastructure, both in the USA and abroad. The current infrastructure in the USA is not sufficient to provide GRFT to the large population of individuals who would potentially benefit from it. Therefore, a further expansion of production infrastructure of current facilities is necessary along with expansion of production into regions heavily affected by HIV. The optimization of production systems for GRFT provides a platform for further economic analysis of the viability of PMPs and comparison of upstream production systems. Although any economic analysis is product and process specific, it is likely that GRFT would represent a best-case scenario for nonvaccine PMPs because of its high expression levels, stability, potency and its current indication for topical delivery – meaning some of the qualification assays may not be as stringent as they are for injectables.

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Conflict of interest

Kenneth Palmer is a member of Intrucept Biomedicine LLC that holds a licence to make and sell Griffithsin from the United States Public Health Service.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 rTMV: GRFT Plasmid Map.